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The intracellular replicating form of T7 DNA is a concatemer in which linear genomes are joined head to tail by sharing 160-bp terminally repeated sequences. A unique hairpin (M-hairpin) end generated on the left side of the TR was proposed to be responsible for the duplication of the concatemer junction for efficient packaging. We characterized the defects caused by loss of the M-hairpin by constructing a recombinant T7 ($T7_{\Delta m}$) deleted in the *m* region. Initially, the intracellular growth rate of progeny phage was normal in $T7_{\Delta m}$ infection. However, the titer of progeny phage was eventually reduced by two- to threefold and lysis was significantly delayed. The restriction fragment, $LE_{\Delta 160}$, generated simultaneously with the double-strand cleavage at the onset of packaging reaction was found more or less at the same intensity in both $T7^+$ and $T7_{\Delta m}$ infection at the beginning but preferentially in $T7_{\Delta m}$ infection during the later phase of infection. These observations suggest that the DNA packaging of T7 proceeds on the intact concatemer junctions during the early stage of infection while the duplication of the concatemer junction by the M-hairpin seemed to be important during the later phase, presumably due to reduced replication. While the generation of the M-hairpin involves DNA replication, the loss of *m* did not reduce DNA synthesis, suggesting that the role of the M-hairpin as an origin of replication is minimal.

INTRODUCTION

The genome of bacteriophage T7 is an approximately 40-kb linear double-stranded DNA with a 160-bp identical sequence at each end (Dunn and Studier, 1983). Upon infection, the linear genomes are replicated and joined head to tail by sharing the terminally repeated sequence (TR) (Watson, 1972; Langman *et al.,* 1978). These concatemers are huge molecules containing hundreds of copies of T7 genomes (Paetkau *et al.,* 1977). During the later stages of infection, the concatemers are processed, coupled to a packaging reaction regenerating the two ends of the shared TR (Kelly and Thomas, 1969).

The orientation of T7 DNA is usually referred to as left and right because all the transcripts face in one direction, to the right (Dunn and Studier, 1983). The fusion of the right and left ends via shared TR brings up two potential genetic elements, *pac*R and *pac*L, which are the sites at which the mature ends are regenerated by the packaging complex (Chung and Hinkle, 1990b). The *pac*R site where the mature right end is generated is composed of sequences from the right-hand part of the TR and sequences next to the TR on the genetic left end. The *pac*L site for the left end is similarly constructed on the left-hand border region of the TR.

The processing of the T7 concatemers is sequential in that the right end is generated by a double-strand cleavage ahead of the left end and the packaging proceeds from right end to left end. The frequency of recombination of markers dispersed along the genome and, more recently, DNase-protection experiments showed that the packaging proceeds leftward (Roberts *et al.,* 1978; Son *et al.,* 1993; Khan *et al.,* 1995). On the intracellular concatemer DNA, right ends were detected exclusively because the generation of a left end releases the packaged genome from the concatemer complex (Chung *et al.,* 1990).

The sequential processing of ends by double-strand cleavage on each concatemer junction makes the unused site unavailable for the other reaction. For example, the generation of the right end at *pac*R makes the *pac*L on the other side of the TR unavailable for the generation of the left end for proper termination of packaging started at an adjacent concatemer junction. Either T7 wastes a fair amount of its replicated DNA or the concatemer junction has to be duplicated.

A peculiar restriction fragment seemingly related to the duplication of the concatemer junction was observed both in an *in vitro* reconstitution experiment and in digestion of the intracellular DNA (White and Richardson, 1987; Chung *et al.,* 1990). One end of the restriction fragment was located at 99.1% of the genome regardless of the restriction enzyme employed and the end was found actually to be a hairpin (M-hairpin) arising out of a 41-bp incomplete palindrome (*m* site) (Chung *et al.,* 1990). Because the companion fragment expected from a double-strand cleavage of the cruciform was not detected, the fragment was thought to be generated by a nick and subsequent rolling-circle-type replication. The double-strand cleavage in the middle of the genome would be obviously detrimental to the phage and such unidirectional replication implicates the evolution of an elaborate

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mechanism for a specific purpose. It is likely to be the duplication of the concatemer junction and/or a new origin of replication.

The best opportunity to test the role of the M-hairpin could be obtained by examining the intracellular DNA after infection with T7 deprived of the *m* site ($T7_{\triangle m}$). We obtained a deletion mutant based on the recently developed protocol to screen mutant phages deleted in functions which are nonessential but required for optimal growth (Kim and Chung, 1996). For quantitative analysis, the gel-embedding technique was applied to recover the intracellular DNA.

MATERIALS AND METHODS

Bacterial strains and bacteriophages

Escherichia coli B and wild-type T7 phage were from F. W. Studier (Brookhaven National Laboratory).

Chemicals and enzymes

Restriction enzymes and modifying enzymes were purchased from Promega. Digoxygenin labeling kit was from Boehringer Mannheim. Agarose was obtained from FMC and other chemicals were purchased from Sigma and Fluka. LumiPhos 530 was provided by Promega and [^{3}H]-thymidine was from Amersham.

One-step growth experiments

The general procedure for the one-step growth experiment was described before (Kim and Chung, 1996). To determine the titer of the intracellular phage, 1 ml of appropriately diluted infection mixture was taken at various times and vigorously mixed with 1 ml of chloroform. At the end of a series of sampling, 0.1 ml of aqueous phase separated from the chloroform layer was taken and mixed with 0.2 ml of overnight-grown *E. coli* cells and plated on LB agar.

Preparation of agarose gel-embedded intracellular DNA

E. coli B was grown with vigorous agitation at 30° to 1.5×10^8/ml and infected with the phage at a multiplicity of infection of 15. At appropriate times after infection, 5 ml quenching solution (2% (w/v) phenol, 8 m*M* EDTA, 95% ethanol, 0.02 *M* sodium acetate (pH 5.4)) (Paetkau *et al.,* 1977) was added to an equal volume of the sample. The pellets obtained by centrifugation at 5000 rpm for 10 min in JA17 rotor (Beckman) were resuspended in 500 μl TNE (10 m*M* Tris–HCl (pH7.5), 50 m*M* NaCl, 1 m*M* EDTA) equilibrated at 47° and mixed with the same volume of 1% low-melting-temperature agarose at the same temperature. The melted agarose-sample mixture was sucked into Tygon tubing (ID = 1/8 in.) and left to solidify on an ice bath. The sample embedded in the gel was subsequently treated with 5 to 8 vol of lysis buffer (10 m*M* Tris–HCl (pH 8.0), 100 m*M* EDTA, 1% SDS, 100 μg/ml proteinase K) overnight at 50° and then again at 37°. The sample was then washed three times with 10 vol of the storage buffer (10 m*M* Tris–HCl (pH 8.0), 10 m*M* EDTA, 1 m*M* PMSF (phenylmethylsulfonyl fluoride)) at room temperature for 60 min and three times with 10 vol of 10 m*M* Tris–HCl (pH 8.0) for 30 min.

For subsequent experiments, a suitable length of the gel-embedded DNA cylinder was sliced off and equilibrated by soaking in 10 vol of restriction enzyme buffer for 30 min. The buffer was decanted and the equilibrated gel slice was melted by incubating at 60° for 10 min before addition of enzyme. Typically 100 units of *Hae*II and 25 units of *Xmn*I were required for the complete digestion of a slice.

Southern hybridization and luminography

*Hae*II- or *Xmn*I-digested DNA was loaded onto a 1% agarose gel and electrophoresed overnight. The DNA was transferred to a nylon membrane according to the published procedure (Sambrook *et al.,* 1989) and fixed by exposure to UV light in a Spectrolinker XL-1000 UV crosslinker (Spectronics) at 120 mJ/cm^2.

The probe was an 834-bp T7 fragment isolated after digestion of pCJ7 (Chung and Hinkle, 1990b) with *Bam*HI. The probe contained T7 sequence from 39381 to 439 and included genes *19.5, m*, and the TR. About 1 μg DNA was labeled by primer-extension reaction using digoxygenin-dUTP with Klenow enzyme.

Hybridization was carried out at 68°. The membrane was washed once with 2× wash buffer (2× SSC, 0.1% SDS) at room temperature for 5 min and twice with 0.1× wash buffer (0.1× SSC, 0.1% SDS) at 68° for 15 min. Then the membrane was equilibrated and treated with the anti-digoxygenin-antibody-conjugated alkaline phosphatase and the luminography was performed by adding LumiPhos 530 and exposing X-Omat AR film (Kodak) at room temperature as recommended by the manufacturer (Boehringer Mannheim).

Preparation of labeled intracellular DNA

E. coli B was grown in M9 supplemented with casamino acids and thiamin. Immediately before infection, uridine was added to 0.05 mg/ml in a 25-ml culture. The phage particles were added at a multiplicity of infection of 10. For the labeling of replicating DNA, [^{3}H]thymidine was added at 5 min postinfection to 18 μCi/ml. To minimize the loss of DNA during the preparation, phenol extraction was avoided as described by Son *et al.* (1988). At 10, 15, 20, and 25 min, 5 ml of culture was mixed with ice-cold quenching buffer (see above). The cells were pelleted by centrifugation in a JA17 rotor (Beckman) at 4000 rpm for 10 min and resuspended in 50 μl lysis buffer (0.05 *M* Tris–HCl (pH 7.4), 25% sucrose, 0.15 *M* NaCl, 0.005 *M* EDTA, 0.004 *M* KCN). Lysozyme was added to 50 μg/ml and the samples were incubated for 10 min in the ice bucket. The DNA was released from cells and

phage by adding 5 μl of 10% sarkosyl followed by incubation for 30 min on the ice and for 60 min at room temperature. Finally RNaseA was added to 50 μg/ml and followed by 15 min incubation at room temperature.

The incorporated radioactivity was measured by precipitating nucleic acids in 5% trichloroacetic acid–0.02 *M* sodium pyrophosphate solution for 15 min and filtering them with GF/C filters (Whatman). The radioactivity was determined in the presence of 2 ml ScintA-XF (Beckman) in a LS6000TA scintillation counter (Beckman).

RESULTS

Construction and isolation of $T7_{\Delta m}$ phage

pSH11 is a plasmid containing two T7 fragments (Fig. 1a). The larger one includes the right origin of replication (OR) and gene *19.5,* while the smaller one contains the TR with about 50-bp flanking sequences from both ends of the T7 genome. Together the two fragments constitute a concatemer junction deleted 168 bp, including the 41-bp *m* palindromic sequence.

When T7 infects *E. coli* carrying pSH11, two additional types of particles could be expected other than T7 particles. Because the cloned concatemer junction is fully functional, the initiation of packaging on pSH11 results in a transducing particle in which the plasmid DNA in concatemeric form is packaged instead of the T7 DNA (Chung and Hinkle, 1990a). Some of the initiations on pSH11 still package largely T7 DNA because the right origin region is highly recombinogenic (Kim and Chung, 1996). The resulting $T7_{\Delta m}$ particles contain a complete T7 sequence except the very right end, which is from pSH11 and thus without the *m* region (Fig. 1b).

We showed earlier that T7 mutants deleted in genetic elements required for optimal growth but still dispensable formed small plaques due to the reduced number of progeny phage particles (Kim and Chung, 1996). By assuming that the M-hairpin is such a dispensable but required element, we searched for slowly growing plaques. We were able to find a few plaques reaching average diameter 0.78 mm compared with the majority of plaques reaching average diameter 1.34 mm after 5-hr incubation at 37°. One of them showed reduced size for the right-end fragment by about 170 bp upon *Sca*I-digestion compared with the wild-type T7 DNA, which is consistent with the recombination scheme shown in Fig. 1b to yield $T7_{\Delta m}$ (data not shown). The restriction digestion of $T7_{\Delta m}$ with *Eco*RI, whose site is not found in T7 DNA, confirmed that the right end in $T7_{\Delta m}$ originated by recombination between the T7 inserts in pSH11 (Fig. 1c).

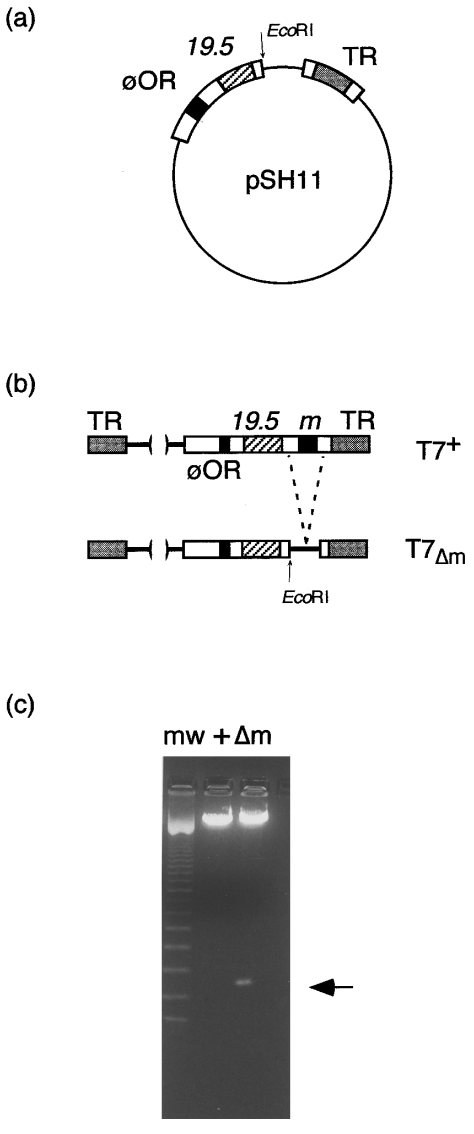

FIG. 1. Construction of $T7_{\Delta m}$. (a) pSH11 is based on pUC19 and contains two T7 fragments. The larger one, spanning from 38981 to 39550 of T7 DNA, includes the right origin of replication (depicted with the origin-associated T7 RNA polymerase promoter øOR) and gene *19.5* (hatched box). It was derived from pDM85 (Chung and Hinkle, 1990a) and was cloned at the *Eco*RI site of pUC19. The smaller fragment corresponds to the T7 sequence from 39719 to 207 and represents the joint region of the T7 concatemer. It was derived from pCJ17 (Chung and Hinkle, 1990b), contained a copy of the TR, and cloned at the *Bam*HI site. (b) $T7_{\Delta m}$ is deleted for 168 bp (from 39551 to 39718) including *m*. It was generated by recombination between T7 DNA and plasmid pSH11 *in vivo.* The strategy for the construction of mutant T7 by recombination and screening was described earlier (Kim and Chung, 1996). The unique *Eco*RI site derived from the vector is indicated with an arrow. (c) The DNA was extracted from purified phage particles and digested with *Eco*RI. Unlike wild-type T7 (+), $T7_{\Delta m}$ (Δm) showed a 255-bp fragment (arrow) upon *Eco*RI digestion. mw, molecular weight marker 100-bp ladder (Pharmacia).

Reduction of the burst size by *m*-deletion

The growth of $T7_{\Delta m}$ phage was compared with the wild-type phage by one-step-growth experiments. After infection, the number of infectious particles was measured and the concentration was plotted against the time of infection on a semilogarithmic scale (Fig. 2a). Lysis in the wild-type infection started at 20 min and the phage titer reached the plateau by 35 min. The ratio of the phage concentration at 43 min over that at 19 min (the burst size) was 153, consistent with usual observations. With the $T7_{\Delta m}$ infec-

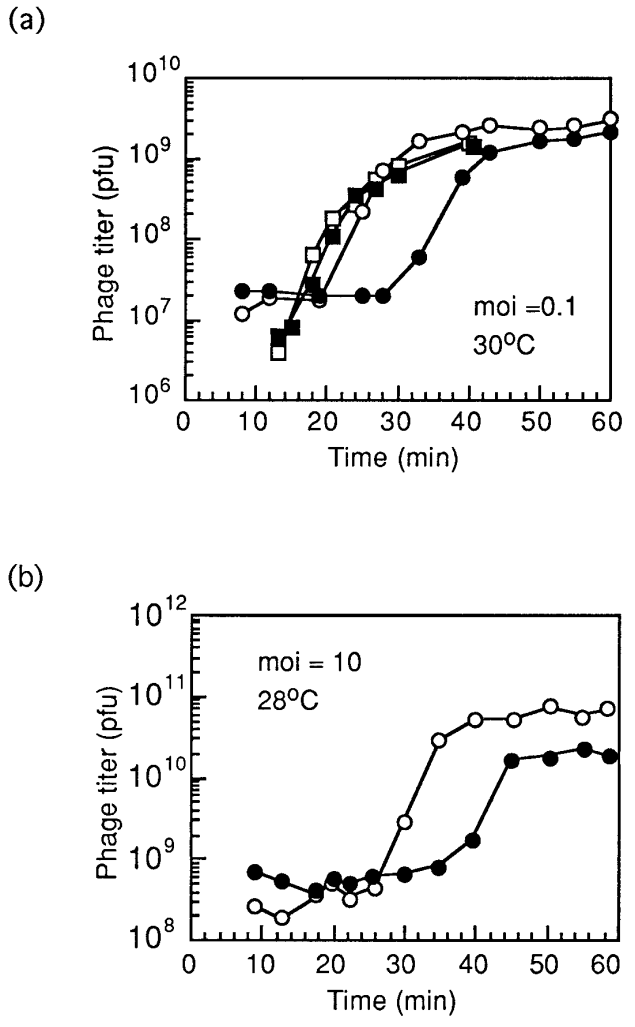

FIG. 2. One-step growth of $T7_{\Delta m}$. *E. coli* was grown at 30° and infected with $T7^+$ or $T7_{\Delta m}$. The concentration of infectious phage was plotted on a log scale against the time after infection. (a) multiplicity of infection (m.o.i.) = 0.1; (b) m.o.i. = 10. PFU, plaque-forming units. Circles represent the phage titer obtained without chloroform treatment while squares are for those with chloroform treatment. Open symbols, wild-type infection; solid symbols, $T7_{\Delta m}$ infection.

tion, lysis started at 30 min and the plateau was reached at 40 min or later. There was a significant delay in the lysis for $T7_{\Delta m}$ infection. The burst size at 43 min was 60, two-and-half-fold reduction from the wild-type infection.

Interestingly, the titers of the internal progeny phage particles observed by lysing cells with chloroform were not significantly different in the two infections at any time (Fig. 2a). This suggests that the production of progeny phage particles was not influenced by the absence of M-hairpin, hence, the duplication of the concatemer junction. The reduction of the burst size in $T7_{\Delta m}$ infection appeared to be caused by the failure of sustained production of progeny phage particles in later stages of infection. Presumably, the duplication of the concatemer junction by the M-hairpin becomes critical for efficient packaging in later stages of infection when the supply of packageable DNA is limited by decline of DNA replication.

The titers of the chloroform-treated samples (squares) are usually much smaller than those of naturally lysed samples (circles) after the lysis of cells, for example, after 40 min. The cause of such reduction in titer has not been understood. However, it could be imagined that chloroform treatment releases from the membrane certain molecules related to phage adsorption to the host cell. These released molecules may bind to the phage particle, particularly to the tail fiber, resulting in the loss of infectivity. Contrary to expectation, the chloroform treatment did not reduce the titer in $T7_{\Delta m}$ infection (Fig. 1a) and this was confirmed by repeated experiments (data not shown). Because the chloroform affects the membrane, the persistent unreduced titer of $T7_{\Delta m}$ phage particles after chloroform treatment suggests that the M-hairpin is somehow related to the disintegration of the cell membrane, as did the observation of the delayed lysis. We do not understand how loss of the M-hairpin could affect the membrane or the timing of lysis. However, another surprising observation made in our laboratory, that a cloned concatemer junction containing *pac*L, *pac*R, and the TR, under a T7 RNA polymerase promoter, severely inhibited T7 growth, suggests that this region is crammed with genetic information important for successful infection (our unpublished observation).

We also carried out a one-step growth experiment at a multiplicity of infection of 10. An identical pattern of infection, namely, reduced burst size and delayed lysis, was also observed at this elevated multiplicity of infection (Fig. 2b).

Accumulation of 160-bp truncated left end in $T7_{\Delta m}$ infection

The concatemer junction is a fused region in which the left and the right end of T7 DNA are joined by sharing the TR. On the concatemer junction, the right-hand part of the TR and a sequence from the left end of the T7 genome form *pac*R, where the right end is regenerated during the packaging, and vice versa (Chung *et al.,* 1990). The initiation of packaging involving a double-strand cleavage at the *pac*R site accompanies the generation of a left end truncated by the length of the TR (Fig. 3a).

The generation of the M-hairpin results in a branched structure whose two left ends are the truncated left end ($LE_{\Delta 160}$) and the M-hairpin end, respectively (Fig. 3a). If the M-hairpin is a structure providing the *pac*L site to save the packaging proceeding toward the truncated left end, loss of the M-hairpin would result in frequent packaging of truncated left ends, making them less labile to exonuclease activity (Fig. 3b) (Sadowski, 1972). The accumulation of $LE_{\Delta 160}$ fragments in $T7_{\Delta m}$ infection could, then, be interpreted as evidence that the packaging proceeds toward the M-hairpin end but not toward the truncated left end.

In Fig. 4a, total intracellular DNAs were examined after digestion with *Hae*II or with *Xmn*I and Southern-hybrid-

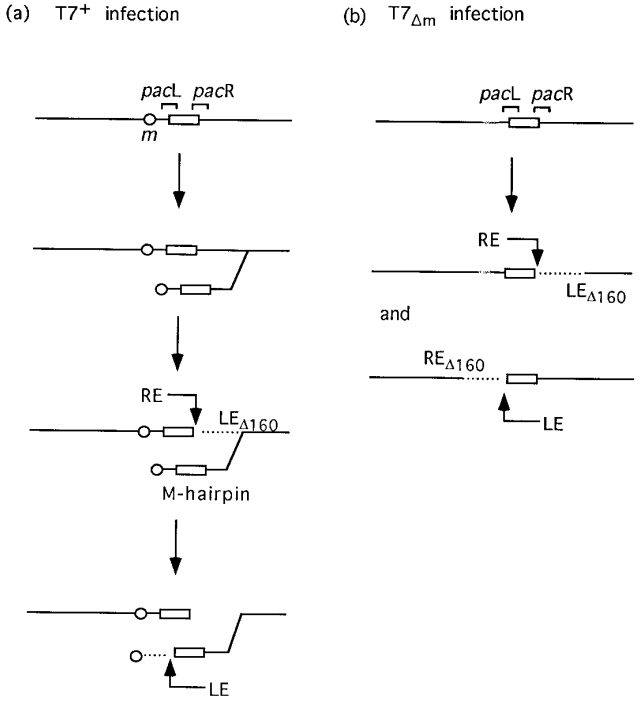

FIG. 3. Schematic diagrams showing processing of concatemer junctions in the presence or absence of the M-hairpin. (a)$T7^+$ infection. *m* (circle), the 41-bp incomplete palindrome where the M-hairpin is generated (Chung *et al.,* 1990). *pac*L and *pac*R, sequences required for the generation of the mature left end and right end, respectively (Chung and Hinkle, 1990b). Each of them consists of part of the TR (rectangle) and flanking sequences as indicated by the square brackets. The M-hairpin duplicates the concatemer junction including the TR. Upon generation of the mature right end (RE), $LE_{\Delta 160}$ ends (dotted line) are produced from the genomic left end region. However, these truncated left ends may not be packaged because gene *3* endonuclease will resolve the branch and the mature left ends should be made at the *pac*L site linked to the M-hairpin. (b)$T7_{\Delta m}$ infection. In the absence of the M-hairpin and, consequently, without duplication of the concatemer junction, the generation of the right end (RE) accompanies production of the left end as in (a). The $LE_{\Delta 160}$ end would be packaged in this case. If the concatemer junction is intact without a preceding initiation event, the termination reaction would accompany the production of truncated right ends ($RE_{\Delta 160}$).

ized with a probe for the concatemer junction region. For the quantitative comparison, we applied the gel-embedding technique, which was originally developed for the recovery of large chromosomal DNA of eukaryotes (Burke *et al.,* 1987). Equivalent numbers of T7-infected *E. coli* cells were harvested at intervals after infection and embedded in agarose gel blocks. Lysis and enzymatic digestions were all carried out within the block with minimal loss of DNA.

We were able to observe bands smaller by about 160 bp than the left end ($LE_{\Delta 160}$) with *Hae*II. Restriction mapping with an additional enzyme, *Xmn*I, demonstrated that the fragments were shorter than the LE by the length of the TR (Fig. 4a). $LE_{\Delta 160}$ was much more abundant in the $T7_{\Delta m}$ infection although it was detected in both wild-type and $T7_{\Delta m}$ infections.

$RE_{\Delta 160}$ is an analogous truncated end to $LE_{\Delta 160}$, generated upon termination of packaging (Fig. 3). $RE_{\Delta 160}$ bands were also observed in both infections and again confirmed by multiple restriction digestion. The $RE_{\Delta 160}$ bands in both wild-type and $T7_{\Delta m}$ samples showed about the

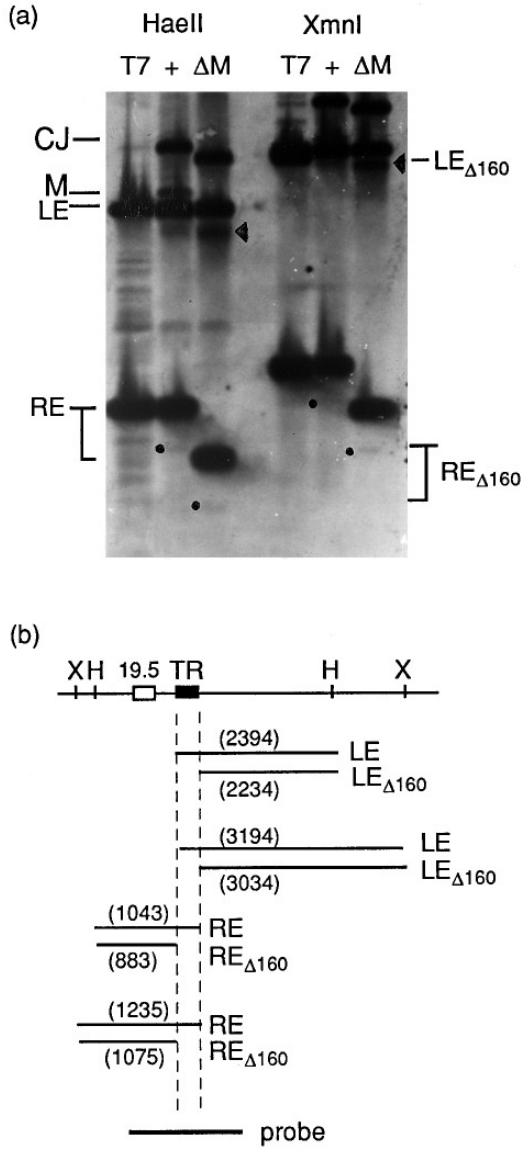

FIG. 4. Accumulation of $LE_{\Delta 160}$. (a) The intracellular DNA at 25 min after infection was digested with either *Hae*II or *Xmn*I and Southern-hybridized with a digoxygenin-labeled probe (a 834-bp fragment corresponding to the concatemer junction from 39381 to 439 of the T7 sequence) depicted in (b). The restriction bands are; CJ, the concatemer junction; M, M-hairpin; LE, mature left end; $LE_{\Delta 160}$, 160-bp-truncated left end; RE, mature right end; $RE_{\Delta 160}$, 160-bp-truncated right end, respectively. "T7," DNA extracted from the purified phage particles; "+," intracellular DNA obtained after infection with wild-type T7; "ΔM," DNA obtained with $T7_{\Delta m}$ phage. $LE_{\Delta 160}$ bands were marked with filled triangles and $RE_{\Delta 160}$ bands were marked with dots. (b) Restriction map for *Hae*II (H) and *Xmn*I (X) in the concatemer junction region. The T7 sequence runs from 1 to 39936 starting from the genetic left end. The *Hae*II sites are from left to right at 38893 and 2394 and *Xmn*I sites are at 38701 and 3194, respectively. The restriction fragments corresponding to the ends are depicted with lines under the map with size (bp) in parentheses. For the sake of brevity, $LE_{\Delta 160}$ is labeled only on the right side in Fig. 4a and other $LE_{\Delta 160}$ bands were indicated with triangles. $LE_{\Delta 160}$ bands were very weak, not being visible in ethidium bromide-stained gels. To detect $LE_{\Delta 160}$, the luminogram in Fig. 4a was over-exposed as is evident from the thickness of the bands.

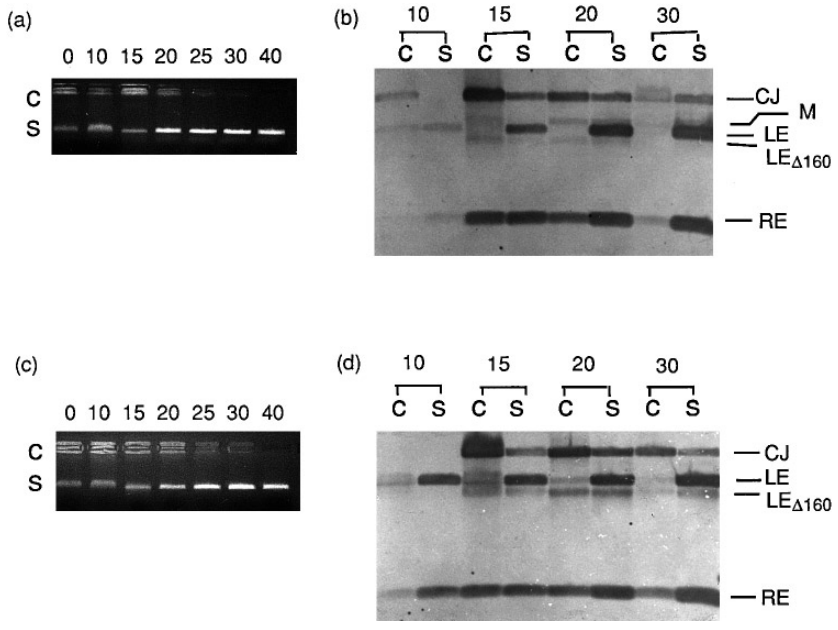

FIG. 5. $LE_{\Delta 160}$ bands in the fractionated intracellular DNA at various times. The intracellular DNAs at indicated times (numbers) were prepared by gel embedding (see Materials and Methods). (a), (b) and (c), (d), $T7^{+}$- and $T7_{\Delta m}$ infection, respectively. (a) and (c) Each lane contained DNA samples from 1.5×10^7 cells. C indicates DNA in the well which does not enter the gel due to its complex structure and S indicates simpler DNA, which is partly degraded *E. coli* chromosomal DNA (up to 10 min) and a mixture of mature T7 DNA released from phage particles and small linear T7 concatemers at later times. Electrophoresis was carried out in a low-melting temperature agarose gel at 80 V for 1 hr. (b) and (d) The part of the gel containing C and S DNA in (a) and (c) was cut out and digested individually with *Hae*II. Each sample was loaded individually and electrophoresed on a new agarose gel as above and hybridized with the probe for the concatemer junction as described in the legend of Fig. 4.

same intensity, unlike the $LE_{\Delta 160}$ band. Although the extreme weakness of the band intensity makes any inferences provisional, the same intensity of $RE_{\Delta 160}$ band in both infections suggests that most of the packaging in the wild-type infection terminated on an intact concatemer junction, i.e., not on the M-hairpin, because $RE_{\Delta 160}$ is generated only when packaging terminates on an intact concatemer junction (Fig. 3). This is consistent with the same rate of growth of internal phage particles observed in Fig. 2. It appears that the M-hairpin is not necessary until the late phase of infection.

Transition of $LE_{\Delta 160}$

We examined the fate of intracellular DNA after fractionation, to investigate the transition of the truncated left end during infection. Rather than resorting to the traditional sucrose gradient for the separation of replicating concatemeric DNA from the packaged DNA, fractionation and digestion were performed by a gel-embedding technique at various times after the onset of replication. The agarose gels in Figs. 5a and 5c show the undigested total intracellular DNA prepared in such a way. Most of the DNA was concentrated in two regions. The DNA that did not migrate out of the sample well on the top (C) corresponds to the topologically complex large DNA sedimenting fast on the sucrose gradient (Chung and Hinkle, 1990a). The single but fuzzy band below (S) corresponds to fragments of *E. coli* DNA still present at early times and linearized T7 DNA at later times when *E. coli* DNA is degraded. There seems to be little difference in the total mass of DNA at any time, comparing the wild-type and $T7_{\Delta m}$ infection. However, it was noted that there is significantly more DNA in the well in $T7_{\Delta m}$ infection, especially between 20 and 30 min.

Each of the bands in Figs. 5a and 5c was separately sliced out and digested with *Hae*II in dilute agarose gel solution. The reactions were loaded onto another agarose gel. The restriction band pattern after staining with ethidium bromide was almost indistinguishable between the wild-type and $T7_{\Delta m}$ infection (data not shown). We observed earlier that the *E. coli* DNA persisted up to 20 min in $T7_{\Delta 19.5\text{-}m}$ infection (Kim and Chung, 1996). The disappearance of *E. coli* DNA in this experiment supports our conjecture that gene *19.5* protein participates in the degradation of *E. coli* DNA.

The gel was hybridized with the same probe used in Fig. 4 to locate the ends and concatemer junction fragments. The C and S of Figs. 5b and 5d indicate the DNA originating from the well and bands as in Figs. 5a and 5c. The infection was carried out at 30° and the replication and lysis normally start at 9 and 20 min, respectively. Few concatemers were observed at 10 min in Fig. 5b. The concentration of the concatemer band of fraction C reached its peak at 15 min, then declined and almost vanished by 30 min. The LE band representing the packaged DNA accumulated gradually to 30 min only

in fraction S. The prevailing RE band in the absence of LE band in fraction C was as observed previously and indicates that the packaging starts with the generation of the right end directly on the complex concatemers (Chung *et al.,* 1990).

The CJ band is also observed in fraction S in Fig. 5b as observed before (Chung *et al.,* 1990). It was suggested that linear but larger than unit-length T7 DNA should be released from the large complex molecules in fraction C by the simultaneous but independent reaction of three activities; the double-strand cleavage by the packaging complex, the generation of the M-hairpin end, and the trimming activity of gene *3* endonuclease (Paetkau *et al.,* 1977; deMassy *et al.,* 1987; Chung *et al.,* 1990). Since fraction S DNA was directly obtained from a single band in Fig. 5a, it further supports the existence of such linear concatemers, one end of which is being packaged while the other end is an M-hairpin. In fact, such long linear molecules were already observed in an electron microscopic study before the discovery of large complex concatemers concentrated in fraction C (Serwer, 1974). The large mass of complex concatemers observed at 15 min in $T7_{\Delta m}$ infection was reduced at later times but, unlike the wild-type infection, the concatemers persist in fraction C even at 30 min (Fig. 5d). Considering that the generation of the M-hairpin is a requirement for the resolution of a looped complex to a linear form, the persistence of large concatemers in these samples is not surprising.

There are relatively few M fragments at 15 min in wild-type infection while the $LE_{\Delta 160}$ bands were found at more or less the same intensity in both infections. This indicates that the packaging occurs mostly on intact concatemer junctions and at the same rate in both infections during the early phase of infection. At 20 min, significantly more $LE_{\Delta 160}$ bands were found in $T7_{\Delta m}$ infection while the M-hairpin develops in wild-type infection. The difference in band intensity of $LE_{\Delta 160}$ in S fractions in Figs. 5b and 5d should reflect the protected $LE_{\Delta 160}$ by packaging in $T7_{\Delta m}$ infection and relatively rapid degradation of $LE_{\Delta 160}$ fragments in the wild-type infection.

The band intensity of $LE_{\Delta 160}$ declined at 30 min in $T7_{\Delta m}$ infection. Perhaps phage particles without proper ends are unstable, releasing the packaged DNA quickly. We searched for phage particles with truncated left ends by end-labeling DNA obtained from phage particles purified on a CsCl gradient, but could not detect any unusual ends (data not shown). In our earlier studies with packageable plasmids, we were able to detect such particles by Southern hybridization but the efficiency of packaging was extremely low, about 1% of the plasmids generating normal ends (Chung and Hinkle, 1990b). In this study involving no such packageable plasmids, the phage particles with irregular ends may be too few to be detected.

Figure 5d shows relatively strong bands for mature ends, LE and RE, even at 10 min in the S fraction. This is probably due to the presence of noninfectious phage particles because DNA replication was not even initiated at 10 min as demonstrated by the absence of a concatemer band in fraction C. T7 phage preparations typically contain about 20–30% infectious particles (Chung and Hinkle, 1990b) but this particular preparation had a higher proportion of inactive phage particles. Consequently, the intensity of LE and RE at 15 min is stronger in Fig. 5d than in Fig. 5b, while they appear more or less similar to each other at later times.

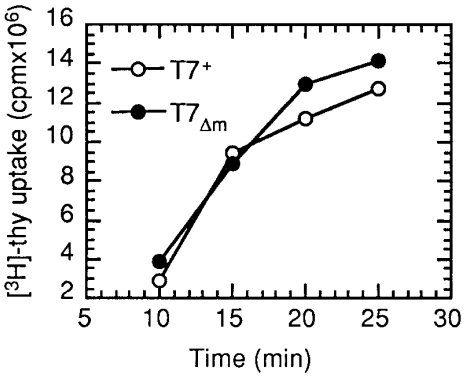

FIG. 6. [^{3}H]Thymidine incorporation. Intracellular DNA was prepared after infection with either wild-type or $T7_{\Delta m}$ phage. Incorporation of the radioactivity for 1.5×10^7 cells was measured by acid precipitation (Sambrook *et al.,* 1989).

In summary, examination of the fractionated intracellular DNA at various times after infection supported the hypothesis developed earlier and in Fig. 3 that packaging occurs on intact concatemer junctions during the early phase of infection while the M-hairpin is required to obtain the maximum efficiency of packaging during the late phase of infection.

DNA replication of $T7_{\Delta m}$

The proposed mechanism of M-hairpin generation involved a unidirectional DNA replication via a rolling-circle mechanism (Chung *et al.,* 1990). Whether the replication accompanying the M-hairpin generation is a limited process serving the duplication of the concatemer junction or is a hitherto unknown pathway of T7 replication has not been determined. Although the recovery of intracellular T7 DNA by gel embedding showed little difference, as judged by the intensity of the fluorescence of ethidium bromide, we estimated the extent of DNA replication by labeling with [^{3}H]thymidine. The intracellular DNA was prepared as described by Son *et al.* (1988) to minimize loss of the DNA. The incorporated radioactivity as determined by acid precipitation for various times after infection is shown in Fig. 6. We were not able to discern significant differences between mutant and wild-type infections.

DISCUSSION

Many bacteriophages with double-stranded DNA genomes form concatemers during intracellular growth because the ends of the template strand cannot be repli-

cated completely by the lagging strand synthesis mechanism. Bacteriophage T7 generates concatemers by sharing the 160-bp terminal redundancy (TR). Upon initial replication of T7 DNA, the ends are left unreplicated and two daughter molecules undergo dimerization via the complementary base-pairing at the ends (Watson, 1972; our unpublished results). The generation of concatemers via the shared TR poses the burden of regenerating two ends with TR at the end of infection. Three scenarios can be imagined for T7: (1) a pair of staggered nicks across the terminal repeat sequence as seen in phage lambda (Feiss and Becker, 1983) and subsequent strand-displacement replication (Kelly and Thomas, 1969); (2) double-strand cleavage for both ends. The packaging could be successful in this case only if the two flanking TR are processed properly, one for the right end and the other for the left end, and the DNA between the two successfully packaged genomes will be wasted. This scenario would be plausible only if packageable DNA is far in excess of the packaging capacity. (3) Double-strand cleavage but with duplication of only the concatemer junction region.

While the first model was perceived to be likely by analogy to the processing of the *cos* site in lambda, *in vitro* studies with cell extracts showed that the packaging reaction accompanied a double-strand DNA cleavage, producing a mature right end and favoring the latter models (White and Richardson, 1987). Restriction digestion of fractionated intracellular T7 DNA also demonstrated the generation of the mature right end ahead of the left end, supporting the double-strand cleavage and sequential generation of ends (Serwer *et al.,* 1987; Chung *et al.,* 1990).

The second scenario looks wasteful and inefficient, making it an unlikely mechanism. However, the experiments shown in Fig. 2 demonstrated indistinguishable rates of growth for wild-type and $T7_{\triangle m}$ phage during the early phase of infection. This indicates that packaging does not require duplication of the concatemer junction, at least during the early phase of infection. In fact, $LE_{\triangle 160}$ was found at nearly the same intensity in both infections at 15 min (Fig. 5), suggesting that the initiation of packaging occurs without the M-hairpin during the early stage of a wild-type infection. Presumably, the supply of T7 DNA exceeds the requirement for packaging during the early phase of infection, so that most of the packaging reactions occur on intact concatemer junctions.

The second scenario appears wasteful because the DNA between the two concatemer junctions utilized for initiation could not be packaged and because any two initiations on adjacent concatemer junctions would lead to an abortive packaging due to the unavailability of the *pac*L site. However, we realized that an economy of packaging could be achieved if the replication forks do not move far into the next genome copy. In this context, the observation by Paetkau *et al.* (1977), that the major intracellular replicating structure has many loops emanating from a dense core and the average length of a loop is close to the unit length of T7 genome, is very suggestive.

The M-hairpin was a nice solution to the third scenario. The hairpin is generated asymmetrically very near the right end with the hairpin end on the left side of the concatemer junction, but not the other way. This suggests that the cruciform, the precursor of the M-hairpin, is nicked at a specific sequence followed by a strand-displacement replication reminiscent of the rolling-circle type of replication. Nicking should be similar but not identical to the resolution of Holliday structures for homologous recombination (Holliday, 1964). Our experiments in Figs. 2 and 5 demonstrate that the M-hairpin is important only during the late phase of infection.

The model in Fig. 3 implicitly assumes the packaging of already fully replicated DNA. In other words, the M-hairpin and partial duplication of the concatemer junction could be useful if the replication is finished and concatemers are ready to be packaged. During the early phase of infection when bidirectional replication is at its peak, generation of the M-hairpin is likely to be a burden rather than a rescue. Thus, the M-hairpin shows up when replication declines and the templates rather than the replicated DNA are ready to be packaged, i.e., during the late phase of infection.

Nothing is known about the mechanism of M-hairpin generation at present. The gene *3* endonuclease has been known to have a specificity for a site like the Holliday junctions generated during recombination (deMassy *et al.,* 1987). Because the nicking site for M-hairpin generation is very similar to the Holliday junction, gene *3* protein was the most likely candidate for the initiation of M-hairpin generation. However, an *amber* mutant in gene *3* clearly showed the existence of the M-hairpin (Chung *et al.,* 1990). T7 has been known to encode another endonuclease whose gene has not been identified yet (Center, 1972). We suggested that gene *19.5* might be responsible for the second endonuclease because the breakdown of *E. coli* DNA was delayed when gene *19.5* was deleted (Kim and Chung, 1996). The location of gene 19.5 at the extreme right end of the genome, which should be expressed last, provides a second clue for the involvement of gene *19.5* in the generation of the M-hairpin.

The third scenario, involving partial duplication of the concatemer junction, may be a feature unique to T7 and its close relatives T3 and K11, since no other bacteriophage with a double-stranded DNA genome seems to share this strategy (Black, 1989). The packaging of bacteriophage T4 appears to start at any point by double-strand cleavage and packages until the head is full (Kalinsky and Black, 1986). In the case of lambda, the concatemer junction is processed to generate both left and right ends with single-stranded regions by using a pair of staggered nicks (Catalano *et al.,* 1995). It is interesting that these phages with great similarity in many aspects such as the morphology, the requirement of terminase composed of one small and one large subunit, and the screwing-in strategy of DNA packaging into the capsid, evolved

different ways of concatemer generation and processing (Earnshaw and Casjens, 1983; Black, 1989).

Although we started this investigation to find $LE_{\triangle 160}$ fragments, the most prominent observation made on $T7_{\triangle m}$ infection was the delayed lysis (Fig. 2). It is not clear how the loss of the M-hairpin seemingly functioning *in cis* can influence the timing of lysis. We recently found that the TR region cloned under a T7 RNA polymerase promoter could severely suppress the growth of T7 (our unpublished result). Interactions around the concatemer junction appear to be far more complicated than originally perceived (Chung and Hinkle, 1990b) and the loss of *m* may indirectly influence the expression of activities encoded in or acting on the concatemer junction region.

The M-hairpin could be an origin for rolling-circle-type replication (Gilbert and Dressler, 1968). The effect of deletion of the M-hairpin should then be observed by changed intracellular mass of T7 DNA. The intracellular DNA prepared by agarose gel embedding is fairly quantitative and ethidium bromide staining did not reveal any detectable difference between the two infections. We also investigated the incorporation of [^{3}H]thymidine (Fig. 6) but again there was little distinction between wild-type and $T7_{\triangle m}$ infection. This does not exclude the possibility of compensation for the lost function, often observed in nature. For example, T7 grows well without the primary origin (Tamanoi *et al.,* 1980). Secondary origins, particularly at 4% from the genetic left end, take up the role of the primary origin in the initial stages of replication. The conclusion that the M-hairpin functions during the late phase of infection when replicating activity declines further supports the idea that the M-hairpin is not responsible for a major replication activity. Generation of the M-hairpin may provide an explanation for the occurrence of two forms of T7 DNA polymerase (Fischer and Hinkle, 1980; Engler *et al.,* 1983). Form II, purified in the presence of EDTA, has high single- and double-stranded DNA exonuclease activity and consequently cannot catalyze strand displacement synthesis at a nick (Lechner *et al.,* 1983). However, Form I T7 DNA polymerase, purified in the absence of EDTA, has 20-fold lower levels of exonuclease activities and this form can initiate limited synthesis of several hundred nucleotides at nicks (Lechner and Richardson, 1983). Although there is little evidence for the existence of two such forms of DNA polymerase in infected cells, it is tempting to suggest specific roles for the two forms of T7 DNA polymerase, Form I being involved in the limited duplication of the concatemer junction.

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